Enteropathogenic *E. coli* translocated intimin receptor, Tir, interacts directly with α-actinin

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Enteropathogenic *Escherichia coli* (EPEC) triggers a dramatic rearrangement of the host epithelial cell actin cytoskeleton to form an attaching and effacing lesion, or pedestal. The pathogen remains attached extracellularly to the host cell through the pedestal for the duration of the infection. At the tip of the pedestal is a bacterial protein, Tir, which is secreted from the bacterium into the host cell plasma membrane, where it functions as the receptor for an EPEC outer membrane protein, intimin [1]. Delivery of Tir to the host cell results in its tyrosine phosphorylation, followed by Tir–intimin binding. Tir is believed to anchor EPEC firmly to the host cell, although its direct linkage to the cytoskeleton is unknown. Here, we show that Tir directly binds the cytoskeletal protein α-actinin. α-Actinin is recruited to the pedestal in a Tir-dependent manner and colocalizes with Tir in infected host cells. Binding is mediated through the amino terminus of Tir. Recruitment of α-actinin occurs independently of Tir tyrosine phosphorylation. Recruitment of actin, VASP, and N-WASP, however, is abolished in the absence of this tyrosine phosphorylation. These results suggest that Tir plays at least three roles in the host cell during infection: binding intimin on EPEC; mediating a stable anchor with α-actinin through its amino terminus in a phosphotyrosine-independent manner; and recruiting additional cytoskeletal proteins at the carboxyl terminus in a phosphotyrosine-dependent manner. These findings demonstrate the first known direct linkage between extracellular EPEC, through the transmembrane protein Tir, to the host cell actin cytoskeleton via α-actinin.

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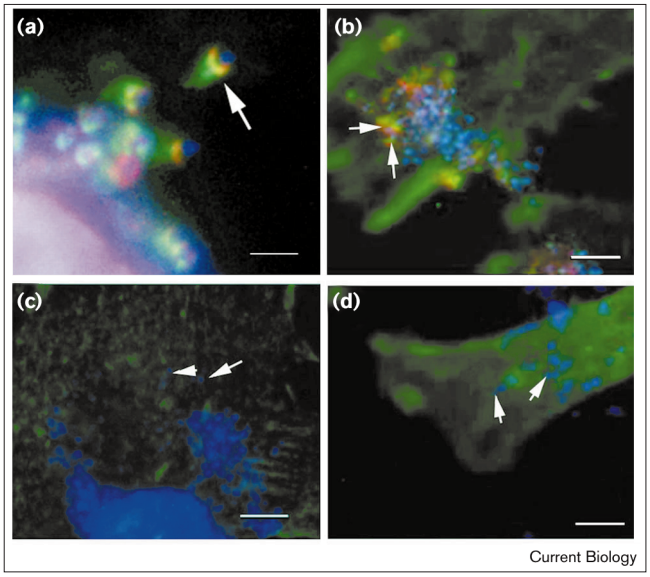
Results and discussion

α-Actinin is recruited to the pedestal in a Tir-dependent manner

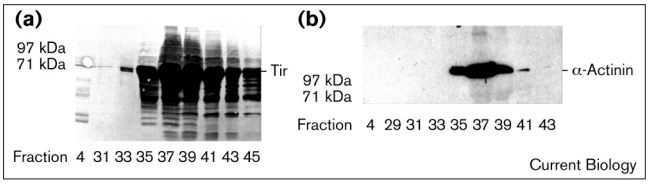
Delivery of Tir to the host cell by the type III secretion system of EPEC is the first step in restructuring the actin cytoskeleton to form attaching and effacing lesions. To determine whether α-actinin recruitment to the pedestal was Tir-dependent, HeLa cells were infected for 5 hours with wild-type EPEC, the intimin mutant CVD206, the Tir mutant Δ*tir*, and a type III secretion mutant cfm14-1-1 (Figure 1a–d). α-Actinin was recruited beneath the wild-type bacterium along the length and at the tip of the EPEC pedestal (Figure 1a). The intimin mutant, which translocates Tir but doesn't cluster it, had an unfocused recruitment pattern of α-actinin beneath the pathogen, which matched the unfocused Tir in the membrane (Figure 1b). The Δ*tir* mutant did not recruit either α-actinin or make pedestals (Figure 1c). The cfm14-1-1 mutant, which cannot secrete Tir, behaved like the Δ*tir* mutant, as no α-actinin was recruited (Figure 1d). HeLa

Figure 1



α-Actinin is recruited to the pedestal in a Tir-dependent manner. HeLa cells were infected with **(a)** wild-type EPEC, **(b)** the intimin deletion mutant CVD206, **(c)** EPEC Δ*tir*, or **(d)** the type III secretion mutant cfm-14-1-1 for 5 h at 37°C and prepared for immunofluorescence. Cells were probed with antibodies against α-actinin (green) or Tir (red) and the nuclear and bacterial DNA stained with DAPI (blue). Scale bars represent 2 μm. Arrows indicate attached bacteria.

Figure 2



α-Actinin elutes with Tir from a His–Tir Ni–NTA column. His-tagged Tir was attached to a Ni–NTA column. HeLa cell lysates were incubated with Tir–Ni–NTA for 1 h, washed extensively, and fractions of increasing imidazole concentrations were western blotted and probed with antibodies against **(a)** Tir and **(b)** α-actinin. Tir elution began at fraction 33 (180 mM imidazole), peaked at fraction 37 (200 nM) (a). α-Actinin eluted at fraction 35 (185 mM), peaked at fraction 37 (200 mM), and slowly eluted until fraction 41 (215 mM).

cells were infected with wild-type EPEC for 1.5, 2, 3, 4, and 5 hours and then prepared for immunofluorescence (data not shown). α-Actinin recruitment to the site of EPEC attachment occurred at 1.5 hours after infection and remained throughout the length of the infection, corresponding with the time of insertion of Tir in the host cell membrane.

α-Actinin elutes with Tir on a Tir affinity column

A Tir affinity column was then constructed and Tir and the associated HeLa proteins were eluted with increasing amounts of imidazole (Figure 2). Tir eluted in approximately the same fractions as α-actinin (Figure 2b). α-Actinin did not elute with the same pattern in the absence of Tir or when His–int282 (the Tir binding domain of intimin) was used (data not shown). These results confirmed the above immunofluoresence data, indicating that α-actinin interacts directly or indirectly with Tir.

Tir binds purified α-actinin directly

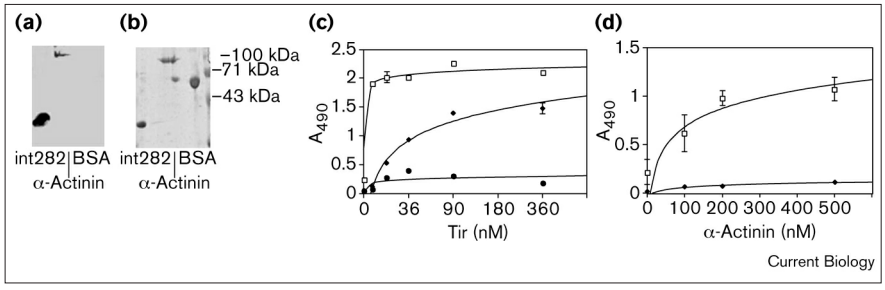
To determine whether Tir binds α-actinin directly, two experimental approaches were used: far-western blotting and enzyme-linked immunoabsorbent assays (ELISAs). In a far-western blot, Tir bound directly to intimin as expected, but also to α-actinin (Figure 3a,b). There was no non-specific binding of Tir to BSA.

To determine whether this binding was concentration dependent, an ELISA was developed. Int282, α-actinin, and BSA were bound to an Immulon-2 96-well plate (0.5 μg/well each) and overlaid with increasing amounts of Tir+CesT (0, 18 nM, 35 nM, 90 nM, 180 nM, and 360 nM per well). CesT is a bacterial Tir chaperone that prevents Tir degradation and aggregation in solution [2]. Tir interacted with α-actinin, saturating at 180 nM Tir (Figure 3c). Int282 also interacted with Tir, saturating at 18 nM Tir. Tir did not bind to the BSA control, nor did it bind non-specifically to the Immulon-2 plate. Purified CesT (350 nM) did not bind int282, α-actinin, or BSA (data not shown). To further establish direct binding, the reverse assay was also performed. Tir+CesT (90 nM/well) was bound to the Immulon plate and overlaid with increasing amounts of purified α-actinin (0, 100 nM, 200 nM, and 500 nM). As shown in Figure 3d, α-actinin bound to Tir directly in a saturatable manner. α-Actinin did not bind non-specifically to the controls. Collectively, the above results show that Tir binds purified α-actinin directly.

α-Actinin binds the amino terminus of Tir

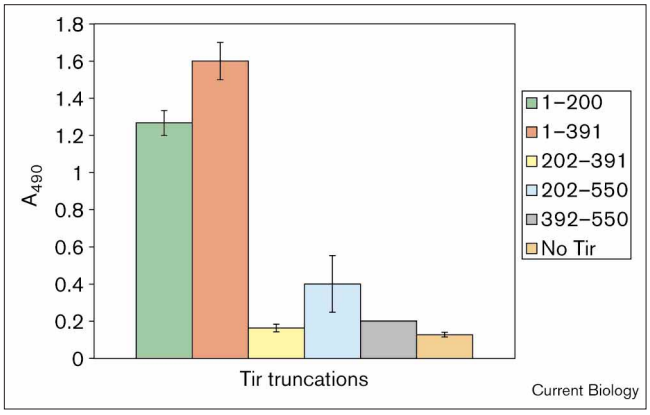
Tir is composed of two transmembrane domains, an extracellular intimin-binding domain (IBD) and two intracellular domains corresponding to the amino and carboxyl termini [3,4]. The carboxyl terminus contains several tyrosine residues, one of which (Tyr474) is phosphorylated in the host cell, a process essential for pedestal formation [3]. To determine which domain of Tir binds α-actinin

Figure 3



Tir binds α-actinin directly. **(a)** Purified α-actinin (Sigma), int282, and BSA (Sigma) were run on an 8% acrylamide gel, transferred to nitrocellulose, and renatured. The nitrocellulose was overlaid with purified Tir for 2 h, then probed with an anti-Tir monoclonal antibody. Tir bound int282 and α-actinin but not to BSA or other high-molecular weight markers. **(b)** Coomassie stained acrylamide gel showing the relative loading of α-actinin, int282 and BSA for the far-western blot in (a). **(c)** Concentration-dependent binding of Tir to α-actinin in an ELISA. α-Actinin (black diamonds), int282 (open squares), and BSA (black circles) were incubated on an Immulon-2 96-well plate and overlaid with increasing amounts of Tir. Tir was detected with an anti-Tir monoclonal and a peroxidase-conjugated secondary antibody. The ELISA shown is a representative of 10 separate experiments done in triplicate. **(d)** Concentration-dependent binding of α-actinin to Tir in an ELISA. Tir (open squares) and BSA (black diamonds) were incubated on a 96-well plate and overlaid with increasing amounts of α-actinin. α-Actinin was detected with an anti-α-actinin monoclonal antibody and a peroxidase-conjugated secondary antibody. The ELISA shown is a representative of four separate experiments done in triplicate.

Figure 4



Tir binds α-actinin through its amino-terminal 1–200 amino acids. ELISA detection of truncated Tir interactions with α-actinin. α-Actinin was incubated on an Immulon-2 96-well plate and overlaid with Tir (1–391), Tir (1–200), Tir (202–391), Tir (202–550) or Tir (392–550). Tir was detected with monoclonal anti-Tir antibodies and a peroxidase-conjugated secondary antibody. The ELISA shown is a representative of seven separate experiments done in triplicate.

directly, truncations of Tir were constructed, purified, and used in an ELISA as described above.

Int282, α-actinin, and BSA were bound to an Immulon-2 plate and overlaid with the various purified Tir truncations. As expected, full length Tir bound to int282 and α-actinin, but not to the BSA control. Tir(1–391), which includes the cytoplasmic amino terminus plus the IBD, bound to both int282 and α-actinin (Figure 4 and data not shown). Tir(1–200), containing only the amino terminus but lacking the IBD, bound α-actinin but not intimin. The cytoplasmic carboxyl terminus of Tir(392–550) did not bind to either protein. Tir(202–550), containing the IBD and the carboxyl terminus, bound to intimin but not to α-actinin. None of the Tir truncations bound to the controls (data not shown). These results demonstrate that the first 200 amino acids of Tir are required for direct binding to α-actinin.

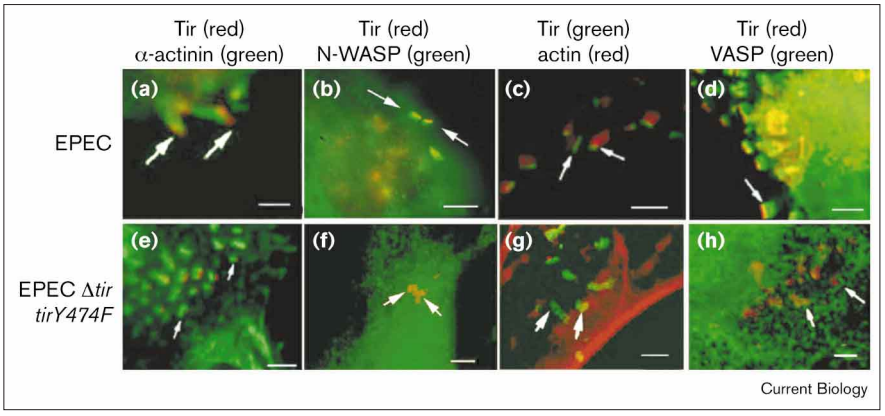
α-Actinin recruitment to the pedestal does not require Tir tyrosine phosphorylation

Given that α-actinin binding occurred within the first 200 amino acids of Tir *in vitro*, the role of tyrosine phosphorylation at the carboxyl terminus (Tyr474) of Tir in the cell was assessed. The EPEC Δ*tir* mutant was complemented with *tir* containing a point mutation (Y474F). This mutant can deliver and focus Tir in the host cell but tyrosine phoshorylation of Tir and pedestal formation does not occur (R.D., unpublished observations). HeLa cells were infected with Δ*tir*/*tir*Y474F for 5 hours, then fixed and prepared for immunofluorescence. Tir was delivered and focused, and α-actinin colocalized with Tir, being recruited and focused beneath EPEC, even without Tir tyrosine phosphorylation (Figure 5a,b). Actin, VASP, and N-WASP were no longer recruited in the absence of tyrosine phosphorylation, despite being focused in the pedestal during wild-type EPEC infection (Figure 5c–h). These results indicate that Tir recruits different cytoskeletal components to the pedestal in a phosphotyrosine independent and dependent manner.

EPEC is among a growing number of pathogens that alter the host actin cytoskeleton during infection. The most well characterized of these systems is *Listeria monocytogenes*, an invasive bacterium that uses actin-based motility (ABM) to propel itself through the host cell cytoplasm (reviewed in [5]). There are several similarites between EPEC and *Listeria* induced actin polymerization events. EPEC recruits similar proteins to its pedestal, including α-actinin, gelsolin (D.L.G., unpublished observations), VASP, and Arp2/3 (reviewed in [6]). Other cytoskeletal proteins, including ezrin, talin, tropomyosin, myosin light chain, villin, T-plastin, and N-WASP are also recruited to the pedestal (reviewed in [6]). Of these proteins, N-WASP and the Arp2/3 complex have been shown to be required for pedestal formation. EPEC acts like *Listeria* in that it

Figure 5

α-Actinin recruitment to Tir is independent of the phosphorylation of Tyr474 of Tir. HeLa cells were infected with **(a–d)** wild-type EPEC and **(e–h)** EPEC *Δtir/tirY474F* and prepared for immunofluorescence. Cells were probed for Tir, α-actinin, N-WASP, actin and VASP. Scale bars represent 2 μm. Arrows indicate areas of Tir delivery (with or without pedestals being formed).



initiates unidirectional actin polymerization beneath the bacterium which may account for EPEC movement on the cell surface [8]. The speed at which EPEC surfs along the membrane, however, is much slower than that of *Listeria*, moving at rates of 0.07 μm/sec [8]. A major difference between EPEC and *Listeria* movement is that there is a plasma membrane separating EPEC and the actin cytoskeleton, unlike *Listeria*, which initiates actin polymerization from within the host cytosol. EPEC requires a ligand on its surface, intimin, and a receptor, Tir, to induce actin polymerization in the host cell in response the extracellular bacterium.

Listeria is commonly studied as a model for actin dynamics, mimicking normal cellular processes like lamellipodia and filopodia formation in migration cells. EPEC may be mimicking another host cell function, focal adhesion formation. Several similarities exist between EPEC pedestals and focal contacts. EPEC mediates binding to the cell across the plasma membrane and remains extracellular throughout the infection. It stimulates outside–in signalling to the host cell cytoskeleton by phosphorylating, binding and clustering Tir via intimin. β1 integrins also transmit signals from the extracellular matrix to the intracellular cytoskeleton by means of ligand occupancy, clustering, and tyrosine phosphorylation. α-Actinin is recruited to focal contacts independently of β1 integrin tyrosine phosphorylation [9], much like its recruitment to EPEC pedestals. It is interesting to speculate that different signalling proteins can be recruited to the EPEC pedestal in response to ligand occupancy, clustering, tyrosine phosphorylation, or a combination of all three. A subset of cytoskeletal proteins, including α-actinin, are recruited independently of the phosphotyrosine while others, including VASP and N-WASP, require Tir tyrosine phosphorylation. A second similarity between EPEC pedestals and focal contacts is that α-actinin binds to β1 integrins directly in focal adhesions and is involved in their organization [10].

A third similarity between pedestals and focal adhesions stems from a high degree of homology shared by intimin and the *Yersinia* outer membrane protein, invasin [11]. Invasin is the ligand on *Yersinia* that mediates its binding to β1 integrins [12]. Invasin–β1 integrin interaction results in the actin-mediated uptake of *Yersinia* into intestinal cells during infection. Structural analysis reveals similarities in the structure of the cell adhesion domains of both intimin and invasin. Furthermore, it has been demonstrated that intimin from EPEC binds β1 integrins [13] although this interaction is not important physiologically [14]. It is interesting to speculate, however, that there may be structural similarities between Tir and β1 integrins that mediate intimin binding and may initiate pedestal formation.

The results presented here give us new insight into how the EPEC pedestal is formed during infection and provide a new role for Tir as a multifunctional protein. Tir not only binds the EPEC ligand intimin but anchors the bacterium directly to the host cytoskeleton. This occurs via a direct linkage through its amino terminus to α-actinin in a phosphotyrosine-independent manner while initiating pedestal elongation in a tyrosine phosphorylation dependent manner. The similarities between the EPEC-induced actin rearrangements and the cytoskeletal changes observed during focal adhesion formation and *Listeria* tail formation suggest that EPEC is a useful tool to study actin dynamics at the plasma membrane.

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